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ORIGINAL PAPER

Combined use of bulked segregant analysis and microarrays reveals SNP markers pinpointing a major QTL for resistance to *Phytophthora capsici* in pepper

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Abstract

Key message **Bulked segregant analysis (BSA) using Affymetrix GeneChips revealed candidate genes underlying the major QTL for *Phytophthora capsici* resistance in *Capsicum*. Using the candidate genes, reliable markers for *Phytophthora* resistance were developed and validated.**

Abstract *Phytophthora capsici* L. is one of the most destructive pathogens of pepper (*Capsicum* spp.). Resistance of pepper against *P. capsici* is controlled by quantitative trait loci (QTL), including a major QTL on chromosome 5 that is the predominant contributor to resistance. Here, to maximize the effect of this QTL and study its underlying genes, an F_2 population and recombinant inbred lines were inoculated with *P. capsici* strain JHAI1-7 zoospores at a low concentration (3×10^3/mL). Resistance phenotype segregation ratios for the populations fit a 3:1 and 1:1 (resistant:susceptible) segregation model, respectively, consistent with a single dominant gene model. Bulked segregant analysis (BSA) using Affymetrix GeneChips revealed a single position polymorphism (SPP) marker mapping to the major QTL. When this SPP marker (Phyto5SAR) together with other SNP markers located on chromosome 5 was used to confirm the position of the major QTL, Phyto5SAR showed the highest LOD value at the QTL. A scaffold sequence (scaffold194) containing Phyto5SAR was identified from the *C. annuum* genome database. The scaffold contained two putative *NBS-LRR* genes and one *SAR 8.2A* gene as candidates for contributing to *P. capsici* resistance. Markers linked to these genes were developed and validated by testing 100 F_1 commercial cultivars. Among the markers, Phyto5NBS1 showed about 90 % accuracy in predicting resistance phenotypes to a low-virulence *P. capsici* isolate. These results suggest that Phyto5NBS1 is a reliable marker for *P. capsici* resistance and can be used for identification of a gene(s) underlying the major QTL on chromosome 5.

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Introduction

Pepper (*Capsicum* spp.) is an economically important vegetable crop worldwide. Pepper production is challenged by various plant pathogens, and developing cultivars exhibiting multiple disease resistance requires time-consuming gene pyramiding processes. The soil-borne pathogen *Phytophthora capsici* L. is the causal agent of root rot, stem rot and foliar blight in pepper (Hord and Ristaino 1991; Kim et al. 1989). *P. capsici* reproduces both sexually and asexually and is a multicyclic disease agent, living on both dead and live plants, as a necrotroph and biotroph, respectively (Bonnet et al. 2007). Under environmental conditions favorable to the pathogen, *P. capsici* can cause up to 100 % yield loss in tropical and subtropical regions. Although resistance to *P. capsici* is affected by the virulence of different isolates (Foster and Hausbeck 2010; Jo et al. 2014), the existence of distinct races remains controversial (Oelke et al. 2003).

Numerous sources of resistance against *P. capsici* have been identified in the cultivated species *C. annuum*, including Criollo de Morelos 334 (CM334), PI201232, PI201234 and AC2258, among which CM334 shows consistently high levels of resistance (Bartual et al. 1991; Oelke et al. 2003). *Phytophthora* resistance is often characterized as polygenic or quantitative, although simple inheritance models have been proposed. The inheritance patterns of resistance against *P. capsici* vary depending on disease screening conditions and isolates of *P. capsici*. For instance, Reifschneider et al. (1992) proposed a two-gene model for resistance after observing resistance inheritance in an F_2 population inoculated with *P. capsici* at 5×10^4 zoospores/mL. By contrast, single dominant gene inheritance was observed when plants were inoculated with 2,000 zoospores/mL (Sy et al. 2005; Walker and Bosland 1999). Indeed, the introduction of resistance into elite susceptible cultivars has been achieved using simple backcrosses in breeding programs of Korean seed companies and many *P. capsici*-resistant hot pepper cultivars are available (Lee et al. 2012), suggesting that the inheritance of resistance can be treated as simple Mendelian genetics for practical breeding applications.

In recent years, research efforts have emphasized identification and mapping of quantitative trait loci (QTLs). QTL analyses have been carried out for *P. capsici* resistance in several intraspecific populations (*C. annuum* × *C. annuum*) by different research groups (Nahm 2001; Ogundiwin et al. 2005; Thabuis et al. 2003), and the results suggest that *P. capsici* resistance is governed by multiple complex quantitative traits. However, the genomic distribution of the QTLs for *P. capsici* resistance is not random. Despite the fact that data are drawn from different populations subjected to different pathogen isolates, disease screening approaches, and scoring methods, there is a strong tendency for QTLs to aggregate, particularly on chromosome 5. Lefebvre and Palloix (1996) identified one major QTL on chromosome 5 explaining 41–55 % of the phenotypic variance among 13 total QTLs detected. Liu (2006) further summarized QTL studies from different research groups for *P. capsici* resistance in pepper (Nahm 2001; Ogundiwin et al. 2005; Thabuis et al. 2003, 2004). The most consistently identified QTLs were those on chromosome 5. Out of 67 QTLs belonging to seven QTL groups according to the original studies, 18 were simultaneously aligned on the middle-to-lower arm of P5 on the integrated pepper map, with five to eleven QTLs located on each of four other chromosomes including chromosome 6, 9, 11 and 12. In most cases, reported LOD scores for 18 QTLs on chromosome 5 ranged from 2.28 to 35.69 (Liu 2006). This observation is consistent with recent QTL analysis (Minamiyama et al. 2007) showing that the QTL located on chromosome P5 accounts for the major contribution to *Phytophthora* resistance (Bonnet et al. 2007; Mallard et al. 2013; Truong et al. 2012).

Despite many QTL studies and identification of the common major QTL for *Phytophthora* resistance, there is no useful marker for marker-assisted selection (MAS) of *Phytophthora* resistance in *Capsicum*. Application of MAS in plant breeding requires markers closely linked to the target locus to be effective for application to a large number of samples and a wide range of crosses in different breeding programs. A sequence-amplified characterized region (SCAR) marker developed from random amplified polymorphic DNA (RAPD) marker OpD04.717 was mapped within 6 cM of the peak of a major QTL located on chromosome P5 in the pepper genetic map AC99 (Quirin et al. 2005). Other *Phytophthora* resistance-linked markers include bacterial artificial chromosome (BAC)-derived markers converted from restriction fragment length polymorphism (RFLP) markers CDI25 and CT211A, targeting QTLs on chromosome P5 and P9, respectively (Kim et al. 2008). However, these markers are not suitable for MAS in practical breeding programs due to lack of polymorphism between breeding lines or low accuracy in predicting resistance phenotypes.

Polymorphic molecular markers based on single nucleotide polymorphisms (SNPs) in DNA sequences are extremely sensitive and can identify individuals within a population. Accordingly, SNPs are believed to represent the most promising marker system for MAS, especially for crop improvement using elite germplasm (Michelmore et al. 1991). The Affymetrix GeneChip microarray provides a high-throughput platform for discovery of single position polymorphisms (SPPs). In this approach, DNA polymorphism is detected from differential hybridization signals of genomic DNA from the tested samples. Bulk segregant

analysis (BSA), which has been widely adopted for rapid identification of molecular makers in specific regions of a genome, is based on the underlying principle that bulking of individuals from a segregating population into pools of alternative phenotypes allows the capture of representative genotypes at a particular locus while a random genetic background is generated at all other unlinked loci (Michelmore et al. 1991). Although the combined use of BSA and microarrays could represent a powerful approach for the discovery of genetic makers, there are only few examples of using these tools together to analyze traits in crop plants due to the high cost and the lack of availability of arrays (Kloosterman et al. 2010; Sherman et al. 2013).

The objectives of this study were: (1) to develop SNP markers tightly linked to the major QTL located on pepper chromosome 5 using a combined BSA and SPP (BSA–SPP) approach, (2) to develop and validate SNP markers applicable for marker-assisted selection of *P. capsici* resistance, and (3) to perform sequence analysis of the major QTL region to reveal candidate genes for contributing to *P. capsici* resistance.

Materials and methods

Plant materials and DNA extraction

The YT population, consisting of 128 recombinant inbred lines (RILs) at the F_8 generation of an intraspecific cross between *C. annuum* YCM334 and Tean, was used for inheritance analysis and marker development (Truong et al. 2012). YCM334, derived from CM334, served as the resistant parent and the Korean landrace Tean as the susceptible parent. Two hundred F_2 segregants were derived from self-pollination of commercial F_1 cultivar *C. annuum* cv. 'Ildangbaek (IDB)' (Syngenta Korea Co., Ltd., Korea). This population was used to develop molecular markers linked to the major QTL for *P. capsici* resistance. The AC99 F_2 population originating from an interspecific cross between *C. annuum* NuMex R Naky (RNaky) and *C. chinense* 'PI159234' (CA4) was used to determine the location of markers (Livingstone et al. 1999). Genomic DNA was extracted with the hexadecyl trimethyl ammonium bromide (CTAB) method from young leaf tissue as described by Yang et al. (2012).

Phytophthora root rot resistance screening and phenotyping

Phytophthora capsici isolate JHAI1-7 was used as inoculum for *Phytophthora* root rot resistance screening. JHAI1-7 was isolated from infected pepper plants in the pepper field in Chungbuk province in Korea and was provided by Dr. Heung Tae Kim. JHAI1-7 is considered to have medium virulence (Jo et al. 2014). To screen for resistance, plants at the six-to-eight leaf stage were tested by applying 5 mL inoculum adjusted to a concentration of 3×10^3 zoospore/mL. Ten plants from each RIL were used. To avoid disease escape, plant materials were grown in 50-hole trays and kept in a greenhouse where the temperature was controlled at 25 °C, with 12 h light/day. Resistance and susceptibility were scored based on severity of wilting at 7–10 days post-inoculation (dpi) using the following index: 0 = symptomless, 1 = mild wilt in leaves or stems (<25 % of wilt), 2 = severe wilt in leaves or stems (<50 % of wilt), 3 = death. The 0 score was classified as resistance and scores 1–3 as susceptibility (Fig. S1).

Microarray analysis

The pepper SNP genotyping array was purchased from Affymetrix (http://www.affymetrix.com/catalog/prod660100/AFFY/Pepper-(Capsicum)-SNP-Genotyping-Array#1_1). A BSA strategy was used to prepare genomic DNA pools for microarray hybridization. For BSA, equal amounts of genomic DNA from 20 resistant lines and 20 susceptible lines selected from YT RILs were bulked, separately. The bulked DNA (30 μg/array) was randomly fragmented with *DNase*I. End labeling of fragmented DNA and hybridization for comparative hybridization were performed according to the modified Affymetrix protocol (Hill et al. 2013). The labeled DNA pools from resistant and susceptible bulks were hybridized on different arrays and hybridization was performed four times for each DNA pool. Probe signals were processed and analyzed based on a non-uniform drop in signal intensity for individual probes within a probe set (Li and Durbin 2009). The R package (http://www.bioconductor.org/) was used to identify SPPs showing a Dstat value of ≥3 or ≤−3 (Borevitz et al. 2003; Gore et al. 2007). EST sequences corresponding to the selected SPPs were identified from the first version *C. annuum* genome database (http://peppergenome.snu.ac.kr).

SNP marker polymorphism survey

A total of 44 SNP markers located on chromosome 5 were used for polymorphism survey between two parental lines, YCM334 and TEAN. First, 17 intron-based SNP markers were surveyed (Park et al. 2014). Primers were designed based on 30 EST sequences containing SPP probes on chromosome 5 (Hill et al. 2013). SNP marker polymorphisms were surveyed by high-resolution melting (HRM) analysis using a Rotor-Gene™ 6000 thermocycler (Qiagen, Germany). PCR was carried out in 20-μL reaction volumes with 50 ng genomic DNA as template, 1× HiPi buffer (ELPIS-Biotech, Korea), 0.2 mM dNTPs, 500 mM

each forward and reverse primers (Bioneer, Korea), 1.5 μM SYTO9 (Invitrogen, USA), 0.6 unit home-made *Taq* DNA polymerase (Desai and Pfaffle 1995). PCR cycling conditions were 95 °C for 4 min, followed by 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s for 45 cycles. Melting curve analysis was performed after PCR termination, and the temperature was ramped from 70 to 90 °C, raised by 0.1 °C per second.

Mapping, linkage and QTL analysis

Single nucleotide polymorphisms (SNP) markers showing polymorphism between the two parental lines were used for linkage and QTL analyses (Table S1). Linkage analysis was performed using CarthaGene software 1.0 (De Givry et al. 2005) and the Kosambi function was used to convert recombination values to genetic distances with a LOD score threshold of 5.0 and a maximum distance of 30 cM. QTL analysis was performed by composite interval mapping (CIM) using QTL Cartographer version 2.5 (Wang et al. 2007) with the forward and backward stepwise regression method. A standard CIM model (8.0 cM walk speed and 5 control markers) was used for control parameters. The threshold value for the QTL significance was determined by permutation test (1,000 replicates) at $P < 0.05$ significance. For detection of QTLs for *Phytophthora* root rot resistance, the calculated threshold of LOD scores was set at 11.5.

Genome analysis

DNA sequences obtained from Phyto5SAR located in the QTL region were used for BLAST (blastn) analysis in the *C. annuum* genome database (http://peppergenome.snu.ac.kr) to identify the corresponding scaffold sequences of the pepper genome. Putative genes were then predicted from scaffold194 sequence using FGENESH (http://linux1.softberry.com) and annotated using BLAST (blastx).

Development and validation of SNP markers using pepper genome sequence

Molecular markers located in scaffold194 were developed for further analysis and validation (Table S1). Four *P. capsici*-resistant accessions (AC2258, YCM334, CM334 and Perennial) and four susceptible accessions (Tean, Jejujaerae, ShinJoGwang and OngGoalChan) were used for sequence comparison to find SNPs between the resistant and susceptible accessions. DNA extracted from each accession was used as a template for PCR. PCR products were cloned into T-Blunt vector (SolGent, Daejeon, Korea) and sequenced at the National Instrumentation Center for Environmental Management (Seoul National University, Korea). The DNA sequences were assembled and compared using the SeqMan program (DNASTAR, Inc., USA). For validation, the developed SNP markers (Phyto5NBS1, Phyto5NBS2_1 and Phyto5NBS2_2) were tested using 100 commercial F_1 hybrid cultivars provided by seven seed companies (NONGWOO BIO Co., Ltd., KOREGON Co., Ltd., Takii Korea Co., Ltd., Sakata Korea Co., Ltd., Syngenta Korea Co., Ltd., Monsanto Korea Co., Ltd. and NONGHYUP R&D Center). For *Phytophthora* root rot resistance screening, four *P. capsici* isolates with different levels of virulence were used. The four *P. capsici* isolates included MY-1 (lowest virulence), KPC-1 and JHAI1-7 (medium virulence), and KPC-7 (highest virulence). *Phytophthora* root rot resistance screening was performed at the Screening Center for Disease Resistant Vegetable Crops (Korea Research Institute of Chemical Technology, Korea). Resistance and susceptibility were scored based on the percentage of plant wilting after inoculation [0–25 % = resistance (*R*), 26–100 % = susceptibility (*S*)].

Results

Screening and phenotyping for *Phytophthora* root rot resistance

Classical inheritance studies indicated that *P. capsici* resistance sources in pepper are polygenic; however, single-gene, two-gene and three-gene-models have been also proposed (Reifschneider et al. 1992; Sy et al. 2005; Walker and Bosland 1999). In this study, we hypothesized that *Phytophthora* resistance in pepper acts as a monogenic trait with resistance being dominant over susceptibility under low disease-pressure conditions, for which we utilized a low intensity inoculation strategy with the *Phytophthora* concentration adjusted to 3×10^3 zoospore/mL. A qualitative phenotyping method was used where index 0 (without any symptoms) was classified as resistance (*R*) and disease indexes of 1, 2 and 3 were classified as susceptibility (*S*). A total of 200 plants from an F_2 population derived from a commercial F_1 hybrid pepper (Ildangbake) were subjected to *Phytophthora* root rot screening, resulting in a segregation ratio of 149:45 (3.3:1, *R* to *S*), which fits a 3:1 segregation model ($X^2 = 0.3367$, $P > 0.05$; Fig. 1a). A segregation ratio of 65:78 (1:1.2, *R* to *S*) was obtained for resistance screening of the YT RIL population. This ratio fits a 1:1 segregation model ($X^2 = 1.1818$, $P > 0.05$; Fig. 1b). These results indicate that under low disease-pressure conditions, the inheritance of *Phytophthora* root rot resistance in pepper follows a simple Mendelian inheritance pattern in the tested populations, behaving as a single dominant trait.

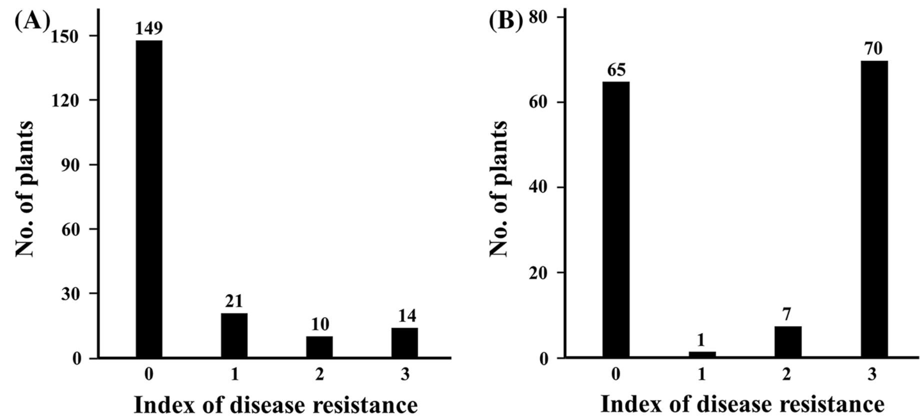


Fig. 1 Frequency distribution of resistance to *P. capsici* strain JHAI1-7 displaying medium virulence. The phenotype index was scored with resistance (*R*) as 0 and susceptibility (*S*) ranging from 1 to 3. **a** The 'Ildangbaek' F2 population showed a 3:1 (*R*:*S*) segregation ratio. **b** YCM334 × TEAN F_8 RILs showed a 1:1 (*R*:*S*) segregation ratio. The parental lines YCM334 and TEAN show phenotype indexes of 0 and 3, respectively

Bulk segregation analysis using microarrays and marker development

We combined a BSA approach with SPP analysis using microarray technology to develop molecular markers linked to the *Phytophthora* resistance exhibiting simple inheritance described above. RILs derived from progeny of a cross between *P. capsici*-resistant YCM334 and -susceptible TEAN were used for the BSA. Based on a monogenic inheritance hypothesis, the resulting resistant RILs are expected to be homozygous for a resistance allele inherited from YCM334, whereas the susceptible RILs should be homozygous for the susceptibility allele from TEAN. In theory, probes genetically linked to the *Phytophthora* resistance locus are expected to show signal differences between the resistant and susceptible trait pools in BSA–SPP-based microarray analysis. Using this approach, a total of 65 SPPs with Dstat values ≥3 or ≤−3 representing ten different candidate EST-based unigenes were identified (data not shown). Eight of the ten unigenes originated from *P. capsici*, possibly due to contamination during pepper RNA preparation after *P. capsici* infection, and these genes were excluded from further analysis. The two plant-derived EST sequences, CAPS.CONTIG.11101 and CAPS.CONTIG.3667, were annotated as receptor-like protein kinase and SAR82A gene promoter region, respectively. We considered these two genes as candidates to be linked to the *Phytophthora* resistance locus and used them for SNP marker development. We performed BLAST searches against the first version of the *C. annuum* genome database (http://peppergenome.snu.ac.kr) to obtain scaffold sequences. CAPS.CONTIG.11101 matched scaffold CAW8S_Contig049087 and CAPS.CONTIG.3667 matched CAW8S_Contig002466. CAW8S_Contig049087 are located on chromosome 11 (Livingstone et al. 1999), whereas CAW8S_Contig002466 is located on chromosome 5. Since previous QTL studies on *Phytophthora* resistance in pepper indicated that the major QTL is located on chromosome 5 (Quirin et al. 2005; Truong et al. 2012), three SNP markers (7890upSNP_2, SNP002466_2 and Phyto5SAR) were developed using CAW8S_Contig002466 for further analysis. For example, 120-bp DNA fragments containing SPP in CAPS.CONTIG.3667 were obtained from YCM334 and TEAN. In the sequence alignment of the 120-bp DNA fragments, SNPs were found at positions 77, 92 and 93. Based on these SNPs, an HRM marker was developed and named Phyto5SAR (Fig. S2A).

Mapping *P. capsici* resistance-linked SNP markers on chromosome 5

A total of 17 SNP markers located on chromosome 5 were used for QTL analysis. Among these were three markers (7890upSNP_2, SNP002466_2 and Phyto5SAR) derived from CAW8S_Contig002466, nine markers (CONTIG9303, 907783, CONTIG1896, CONTIG1820, CONTIG6473, CONTIG5473, KS14035C05, KS17024G04 and KS17054F01) developed from EST sequences and five intron-based markers (IB1556, IB1558, IB1429, IB1119 and IB834). A total of 128 YT RILs were genotyped using this set of markers, and the results were used to construct a molecular linkage map of chromosome 5. The linkage map length was 131 cM, with 7.70 cM on average between markers (Fig. 2a, b). A QTL peak spanning 18.8 cM flanked by two markers, CONTIG6473 and CONTIG1896, was detected for *P. capsci* resistance. The QTL region contained nine markers (CONTIG6473, 7890upSNP_2, SNP002466_2, Phyto5SAR, CONTIG1820, 907783, IB1556, IB1558 and CONTIG1896) at map positions between 14.8 and 33.6 cM (Fig. 2a, b; Table 1); primer sequences for the markers are shown in Table S1. The LOD scores within the interval ranged from 14.98 to 30.14 (Fig. 2a, b; Table 1). Phyto5SAR, with a LOD score of 30.14 and accounting for up to 67.68 % of the phenotypic variance, was identified as the most closely linked marker to the major QTL for *Phytophthora* root rot resistance in this study (Fig. 2a, b; Table 1).

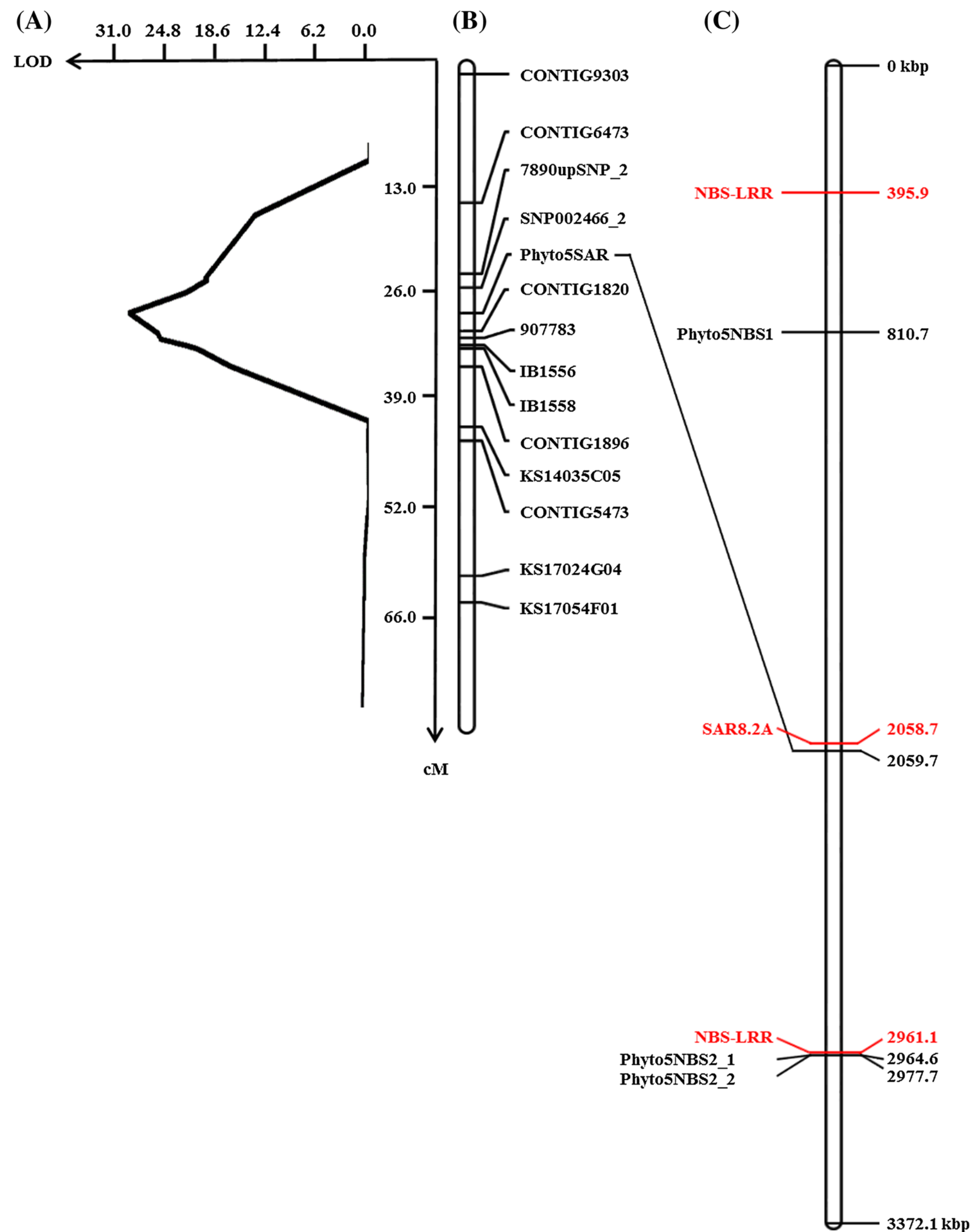


Fig. 2 Local genetic linkage map showing the major QTL and the identification of a scaffold located in the major QTL region. **a** Molecular linkage map and chromosomal locations of the major QTL for *P. capsici* resistance. The linkage map of chromosome 5 was constructed using the YT RIL mapping population. QTLs detected by composite interval mapping analysis are represented by a *solid line*. The LOD scale is shown on *X axis*, while genetic distance (cM) on chromosome 5 is represented on *Y axis*. (B) Genetic map including the major QTL region between markers 7890upSNP_2 and contig1896 (22.9–33.6 cM). The LOD score of Phyto5SAR is the highest. **c** Scaffold194 of CM334 from the *C. annuum* BAC Database (http://peppergenome.snu.ac.kr) in which PhytoSNP5 is located. Newly developed SNP Markers (shown in *black text*; Phyto5NBS1, Phyto5NBS2_1, and Phyto5NBS2_2) co-segregated with Phyto5SAR. Putative resistance genes against *P. capsici*, two *NBS-LRR* genes and *SARS.2A*, are indicated in *red*

Genomic sequence analysis of the major QTL on chromosome 5

Further genomic analysis was carried out for the major QTL interval between markers 7890upSNP_2 and CONTIG1896, covering from 22.9 to 33.6 cM on chromosome 5. This interval region was found to explain more than 45 % of the phenotypic variance in *Phytophthora* resistance (with R^2 ranges from 46.80 to 67.68 %). Sequences of the markers within the region were used as templates to perform BLAST searches in the *C. annuum* genome database to identify the corresponding scaffold sequences. A total of five scaffolds (scaffold194, scaffold1314, scaffold1306, scaffold419 and scaffold619) were identified. Scaffold194 matched the sequences of three markers (7890up SNP_2, SNP002466-2 and Phyto5SAR) showing the highest LOD in composite mapping analysis. This result suggests that target genes for *P. capsici* resistance in the major QTL region may be located within scaffold194. More than 500 putative genes were predicted in scaffold194 by the FGENESH program. Among them, 44 putative genes were predicted to be related to disease resistance, including CC-NBS-LRR class genes [*R2*, *recognition of peronospora parasitica* (*RPP*) 8 and *RPP13*], RLK class genes (*cysteine-rich receptor-like protein kinase 8*), *systemic acquired resistance 8.2A* (*SAR8.2A*) and *AVRRPT2-INDUCED GENE 1* (*AIG1*) (Table S2). However, all candidate genes except two *NBS-LRR* genes and *SAR8.2A* were predicted to be pseudogenes.

Table 1 QTL analysis of resistance to *P. capsici* root rot as detected by composite interval mapping in the YT RIL mapping population

Marker[a]	Position (cM)[b]	Composite interval mapping results		
		LOD[c]	R^2 (%)[d]	Add[e]
CONTIG6473	14.8	14.98	40.55	0.64
7890upSNP_2	22.9	20.31	51.86	0.72
SNP002466_2	24.5	23.07	57.03	0.76
Phyto5NBS1	27.0	30.14	67.68	0.82
Phyto5SAR	27.0	30.14	67.68	0.82
Phyto5NBS2_1	27.0	30.14	67.68	0.82
Phyto5NBS_2	27.0	30.14	67.68	0.82
CONTIG1820	29.5	26.56	62.10	0.79
907783	30.3	26.23	60.63	0.78
IB1556	31.1	22.80	55.55	0.75
IB1558	31.5	21.49	53.59	0.73
CONTIG1896	33.6	17.51	46.80	0.69

[a] Markers located in *P. capsici*-resistance QTL region

[b] Position of the marker in cM on the chromosome

[c] LOD value, the value of the statistical test for QTL detection

[d] R^2 is the proportion of variance explained by a QTL at the associated marker

[e] Additive effect, value representing the additive effect of the QTL expressed in units of a standardized trait

The two *NBS-LRR* candidate genes, located at 395.9 and 2961.1 kb of scaffold194, were predicted to have the complete *NBS-LRR* gene structure with a single exon (Fig. 2c; Table S2). *SRA8.2A*, at 2058.7 kb of scaffold194, and its homologs are highly expressed in *P. capsici*-infected pepper (Lee and Hwang 2003). These results suggest that the two putative *NBS-LRR* genes and *SAR8.2A* are strong candidates for underlying *Phytophthora* resistance.

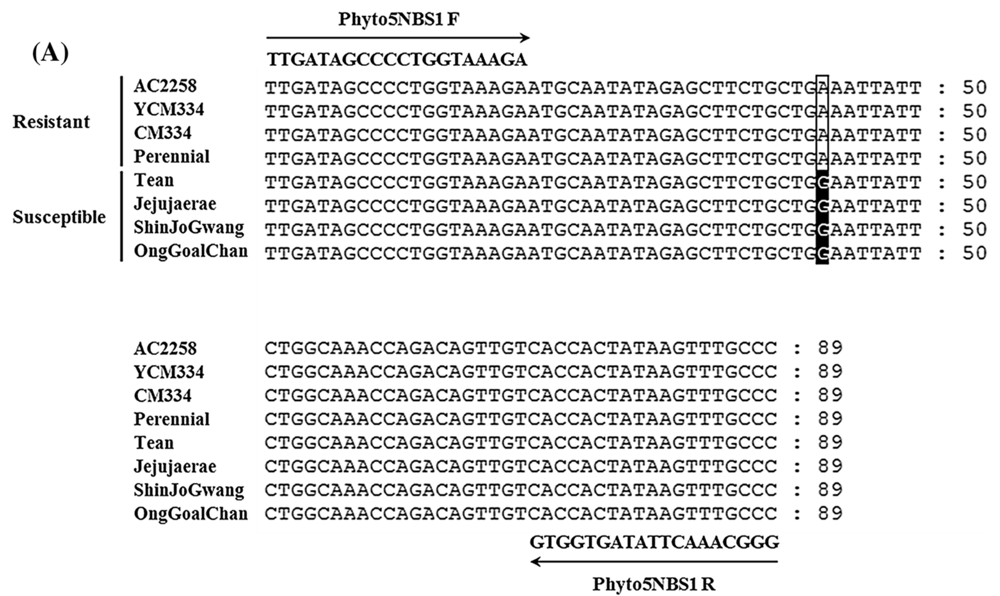
Development and validation of SNP markers for *Phytophthora* root rot resistance

BLAST results against the *C. annuum* genome database indicated that two copies of the Phyto5SAR sequence (Fig. S2A) were present in different scaffolds. To develop single-copy molecular markers using genomic sequences of the major QTL, single-copy sequences were obtained and examined for polymorphisms between the four resistant (AC2258, YCM334, CM334 and Perennial) and four susceptible accessions (Tean, Jejujaerae, ShinJoGwang and OngGoalChan). The Phyto5NBS1, Phyto5NBS2_1 and Phyto5NBS2_2 markers indicated polymorphism between the resistant and susceptible accessions. Phyto5NBS1 contained a SNP (A in resistant and G in susceptible) at position 43 (Fig. 3a), and when Phyto5NBS1 was tested for HRM analysis, the curves for each genotype (resistant, heterozygous, susceptible) were clearly distinguishable (Fig. 3b). Phyto5NBS2_1 indicated two SNPs between resistant and susceptible accessions. The first SNP was used for development of a CAPS marker because only susceptible cultivars had a *Mlu*CI restriction enzyme site (Fig. S2B). Phyto5NBS2_2 indicated SNPs at positions 61 and 64 between resistant and susceptible accessions (Fig. S2C), and HRM analysis using that marker also generated clear curves for each genotype (resistant, heterozygous, susceptible).

To validate these markers for use in monitoring *Phytophthora* root rot resistance, we genotyped 100 commercial F_1 cultivars with the three markers and compared their resistance to *P. capsici* isolates with different virulence (MY-1: lowest virulence, KPC-1 and JHAI1-7: medium virulence, KPC-7: highest virulence) (Jo et al. 2014). Among the 100 F_1 cultivars, cultivars 1–59 are labeled as resistant cultivars (Table S3). In general, phenotypes tested using *P. capsici* isolates exhibiting lower virulence (for example, MY-1) were better matched to the genotypes of the three markers compared with those tested using *P. capsici* isolates exhibiting higher virulence (for example, KPC-7). Among the markers tested, the Phyto5NBS1 genotypes were better matched with resistance phenotypes than were those of Phyto5NBS2_1 and Phyto5NBS2_2 (Table S3). The relationships between the Phyto5NBS1 marker genotypes and *P. capsici* isolates with different virulence are shown in Fig. 4. The Phyto5NBS1 genotyping results showed that 58 cultivars were heterozygous or homozygous for the resistance allele, whereas 42 were homozygous for the susceptible allele (Fig. 4; Table S3). When MY-1 (with the lowest virulence) was tested, 59 cultivars were resistant and 41 cultivars were susceptible. In marker genotype and phenotype comparisons, 91 out of 100 genotypes corresponded to the correct disease phenotype. However, when KPC-1 and JHAI1-7 (with medium virulence) were used, 76 and 73 out of 100 genotypes, respectively, matched the disease phenotype. For the KPC-7 isolate (with the highest virulence), only 54 out of 100 genotypes matched the disease phenotype (Fig. 4; Table S3). These results suggest Phyto5NBS1 is a reliable marker for predicting phenotypes of resistance to *Phytophthora* when less virulent *Phytophthora* isolates are used.

Discussion

Our study establishes the main QTL linked to Phyto5NBS1 on chromosome 5 as a key genomic position in *Capsicum* for resistance to *P. capsici*. We hypothesized that this position may represent the major QTL found in quantitative resistance studies or the major gene identified in classical inheritance studies (Bradshaw et al. 2006; Huang et al.



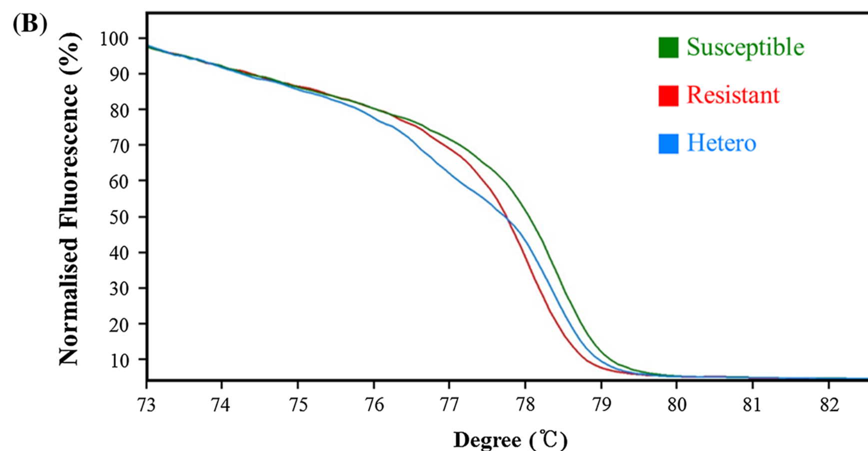


Fig. 3 Sequence and HRM curves of the Phyto5NBS1 marker. **a** Comparison of Phyto5NBS1 sequence from four *P. capsici*-resistant cultivars and four *P. capsici*-susceptible cultivars amplified using the Phyto5NBS1 primers indicated by *arrows*. One SNP found between the resistant and susceptible cultivars is highlighted with a *rectangle*. **b** HRM *curves* for Phyto5NBS1. *Green* susceptible homozygote, *Red* resistant homozygote, *Blue* heterozygote

2005; Reifschneider et al. 1992; Sy et al. 2005). By adjusting the inoculum concentration of a *P. capsici* strain with medium virulence, we were able to obtain 1:1 and 3:1 (*R*:*S*) segregation ratios in a RIL population and F_2 segregating population, respectively. This experimental set-up allowed us to apply a BSA strategy accompanied with SPP analysis using Affymetrix GeneChips, based on the assumption that resistant bulk and susceptible bulk samples contained the resistant and susceptible alleles, respectively, as well as their corresponding linked genomic factors at the target locus. As a result, we were able to develop *P. capsici*-resistance-linked SNP markers by comparing the extracted DNA from resistant and susceptible bulk samples in the microarray analysis.

Our results demonstrate that SNP markers can be widely used for *Phytophthora* resistance breeding programs. SNP markers overcome the major limitation of AFLP- and RAPD-derived markers developed from previous studies, where there was often a lack of polymorphism in working breeding populations (personal communications with breeders). Specifically, we demonstrated that Phyto5NBS1 can clearly distinguish susceptible genotypes from resistant genotypes, allowing flexibility for breeders to introgress *P. capsici* resistance into their materials from different resistance sources. As demonstrated using the commercial breeding materials and F_1 hybrids, Phyto5NBS1 was closely associated with *Phytophthora* root rot resistant/susceptible traits for *P. capsici* strains with low virulence. When *P. capsici* strains with higher virulence were applied, the association between Phyto5NBS1 and *Phytophthora* root rot resistance tended to decline, but the association between Phyto5NBS1 and *Phytophthora* root rot susceptibility remained unaffected. This is likely caused by the increased virulence, which may overcome the resistance conferred by the major QTL linked to Phyto5NBS1. Other QTLs on chromosome 6, 9, 11 and 12 are also important for resistance against *P. capsici* with higher virulence.

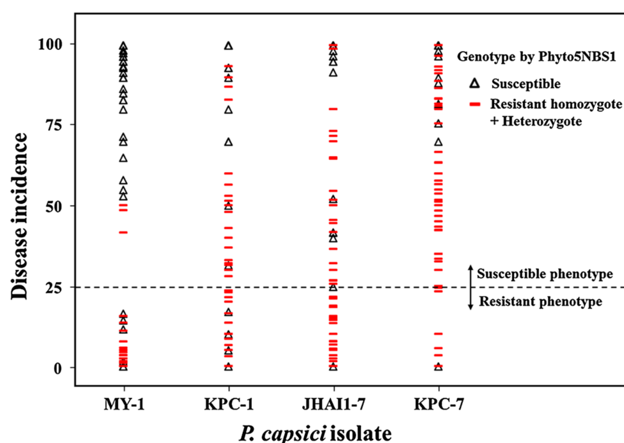


Fig. 4 Validation of Phyto5NBS1 marker by testing 100 commercial F_1 hybrids. *X axis* shows *P. capsici* isolates with different virulence (MY-1: lowest virulence, KPC-1 and JHAI1-7: medium virulence, KPC-7: highest virulence). *Y axis* indicates the degree of disease incidence (from 0 to 100). 0–25 and 26–100 are considered to represent resistance (*R*) and susceptibility (*S*), respectively. For the genotyping with the Phyto5NBS1 marker, *black triangles* indicate susceptible homozygous genotypes and red bars indicate resistant homozygous or heterozygous genotypes

NBS-LRR and RLK type genes are key resistance genes (*R* genes) in plants and are often found in clusters at specific regions (Meyers et al. 2003). The major QTL is found in scaffold194, which is considered to be a disease resistance hotspot where 8 and 34 genes are annotated as NBS-LRR type and RLK type R genes, respectively. However, except for two *NBS-LRR* genes, all of the annotated genes lack functional domains for disease resistance, and are thus considered pseudogenes. The two genes located at 395.9 and 2961.1 kb (Fig. 2c) have intact NBS-LRR structures, suggesting that they are strong candidate genes for *P. capsici* resistance. The association between a major QTL and R genes for *P. capsici* resistance in pepper is not surprising given that many studies have reported that NBS-LRR R genes are observed as QTLs or dominant resistance genes. For instance, the R gene *RB/Rpiblb1* has been demonstrated to be a major QTL accounting for 62 % of the genetic variation in progeny populations derived from a *Phytophthora*-resistant source (Naess et al. 2001; Song et al. 2003). *C. annuum SAR8.2A* is also located within the target locus in scaffold194 (Fig. 2c). *SAR8.2* is a type of systemic acquired resistance (SAR)-related gene and is induced both locally and systemically in pepper plants inoculated with virulent and avirulent strains of *Xanthomonas campestris* and *Pseudomonas fluorescens* (Choi and Hwang 2011), suggesting that *SAR8.2A* is another strong candidate gene for *Phytophthora* root rot resistance in pepper. Recent studies have shown that NBS-LRR-linked and -unlinked loci can function together to confer disease resistance against pathogens. For example, *RPP2A* and *RPP2B* function in *Arabidopsis thaliana* against oomycete isolates, and wheat *Lr10* and *RGA2* confer resistance against fungal pathogen (Eitas and Dangl 2010). Emerging studies also indicate that NBS-LRR proteins and SAR might be functionally associated in plant defence responses (Bonardi et al. 2011). Recently, Rehrig et al. (2014) carried out QTL analysis for *P. capsici* resistance using RILs derived from the resistant accession CM334 and the susceptible accession Early Jalapeño. They positioned the major QTL in the similar region of chromosome 5 as we did. However, the predicted candidate gene was different. They predicted *DOWNY MILDEW RESISTANT 1* homolog as a strong candidate gene. Therefore, we cannot rule out a possibility that more than one gene may contribute to the major effect observed on chromosome 5 as we described above. For the validation of candidate genes in two studies, further works are required.

Previous comparative genetic analysis of disease resistance in the Solanaceae has suggested that disease resistance QTLs and major (typically single dominant) R genes are not randomly distributed in the genomes of pepper, potato (*Solanum tuberosum)* and tomato (*Solanum lycopersicum*) (Grube et al. 2000). In fact, clustering of R genes and QTLs has also been discussed in other plant species including rice (*Oryza sativa*) and maize (*Zea mays*) (Wisser et al. 2005, 2006). R gene clusters typically confer resistance to several unrelated pathogen types. In a previous study, shared genomic locations were not observed for genes that showed similar pathogen specificity, except in the case of *Phytophthora* spp. resistance (Grube et al. 2000). It appears that the genetic structure of chromosome 5 in pepper has undergone complicated chromosomal rearrangement due to translocation through species divergence. The major QTL region on pepper chromosome 5 shows homology to regions on tomato chromosome T4/potato chromosome IV. (Livingstone et al. 1999). This syntenic region in potato chromosome IV contains at least four R genes, including *R2*, and one QTL for *P. infestans* resistance (Grube et al. 2000; Leonards-Schippers et al. 1994; Park et al. 2005a, b, c; Van Der Vossen et al. 2003). Interestingly, the two putative NBS-LRR candidate genes are highly similar to *R2*, *RPP8* and *RPP13*. *R2* in potato and *RPP8/RPP13* in *Arabidopsis* are responsible for resistance to the oomycete pathogens *Phytophthora infestans* and *Peronospora parasitica*, respectively (Lokossou et al. 2009; Rose et al. 2004; Takahashi et al. 2002). The conserved syntenic position and functional characteristics of *Phytophthora* spp. resistance in potato and pepper indicate that the two loci likely arose from the same ancestral sources followed by co-evolutionary processes. The conservation of coding regions among the R gene families might allow the R genes in potato and pepper to recognize the corresponding pathogens *P. infestans and P. capsici*, respectively, while maintaining their syntenic positions (Dangl and Jones 2001; Hulbert et al. 2001; Michelmore and Meyers 1998).

Author contributions WYL and JHK prepared manuscript, and HSJ, HJC, and HBY performed research (sequence 2 analysis and marker development). KKT developed and provided RILs, and KJC screened 3 phytophthora resistance. DC, MJ and BCK participated in its design and coordination of 4 research and helped to draft the manuscript.

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Conflict of interest The authors declare that they have no competing interests.

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